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Award Number: DAMD17-03-1-0717

TITLE: Hormone Replacement Therapy, Iron, and Breast Cancer

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REPORT DATE: November 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2004	Annual (1 Sep 2003 - 31 Aug 2004)

4. TITLE AND SUBTITLE
Hormone Replacement Therapy, Iron, and Breast Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0717

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Hormone replacement therapy (HRT) may increase breast cancer (BC) risk in post-menopausal women. We hypothesize that a high iron level is one of the pre-neoplastic changes in post-menopausal women, and HRT causes iron release to increase BC risk. This hypothesis will be tested using an iron loaded transgenic mouse model. Since iron slowly accumulates due to the mutation of the HFE gene (hemochromatosis Fe), iron elevated in the mouse body mimics the post-menopausal condition. In the present study, we will assess whether (1) HRT mobilizes iron from the liver to the mammary site in the mice, causing greater oxidative DNA and protein damages in the breast tissue; and (2) HRT and iron enhance mammary cancer cell growth. Female wild type and HFE homozygote mice will be fed a diet with or without Prempro™ or inoculated with breast cancer cells into the mammary fat pads and then fed with Prempro™. After treatment, mammary tumor nodules will be counted to determine the tumor incidence. Portions of mammary tissue will be used for assessing oxidative DNA damage and protein oxidation. We expect that HFE homozygote mice subject to HRT will be more susceptible to mammary tumorigenesis than wild type mice.

14. SUBJECT TERMS
Hormone replacement therapy, iron, iron overload, breast cancer, oxidative DNA damage

15. NUMBER OF PAGES
7

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

An estimated 10 million post-menopausal women in the United States currently use estrogen-based hormone replacement therapy (HRT) to ease menopausal symptoms and prevent osteoporosis. Recent epidemiological studies from the Women's Health Initiative indicate that HRT may actually increase the risk of breast cancer (BC), as well as heart attacks, strokes, and blood clots (*Rossouw, et al. 2002*). A meta-analysis of nine prospective studies on post-menopausal levels of endogenous sex hormones and BC showed a strong association of estrogens with BC risk (*Anonymous, 2002*), a rate which is much higher in post-menopausal than pre-menopausal women. Yet, estrogen levels are lower in post-menopausal as compared to pre-menopausal women. In this dilemma, a re-evaluation of the risk factors for BC is necessary. Because of the female baby-boomer generation entering this menopausal status, now there is an even greater need to address BC etiology associated with HRT or estrogens.

In the present study, we hypothesize that a high iron level is one of the pre-neoplastic changes in post-menopausal women, and HRT causes iron release to increase BC risk. This hypothesis will be tested using an iron loaded transgenic mouse model. Since iron slowly accumulates due to the mutation of the HFE gene (hemochromatosis Fe), iron elevated in the mouse body mimics the post-menopausal condition.

Development of iron overloaded transgenic mice:

The murine HFE gene is structurally similar to the human gene. Four different HFE gene disruptions have been reported in the mouse: an exon 4 knockout, an exon 3 disruption/exon 4 knockout, an exon 2-3 knockout, and a C282Y knock-in (*Zhou, et al. 1998, Bahram, et al. 1999, Levy, et al. 1999*). In each model, the mice manifest increased hepatic iron levels, elevated transferrin saturation, and increased intestinal iron absorption. These mice also demonstrate relative sparing of iron loading in reticuloendothelial cells. We have used the exon 4 knockout mice provided by Dr. Nancy Andrews of the Howard Hughes Medical Institute, Harvard Medical School, which are currently bred in our laboratory (*Levy, et al. 1999*). Figure 1 shows the wild type allele and the targeted allele of the HFE knockout mice. Using RT-PCR with primers of HFE Ex3 F: **5'** GTCACGAA GTTGGGA GTGGT **3'**, HFE Int3 R: **5'** CAGCCTTGGCTACAGTGTGA **3'** and HFE Ex5 R: **5'** ATGGTGACCCC ACTG ATGAT **3'**, the designed PCR products of the following two primer sets of HFE Ex3 F/ HFE Int 3 R will be about 500 bp (wild-type) and HFE Ex3 F/ HFE Ex5 R will be 400 bp (HFE homozygote).

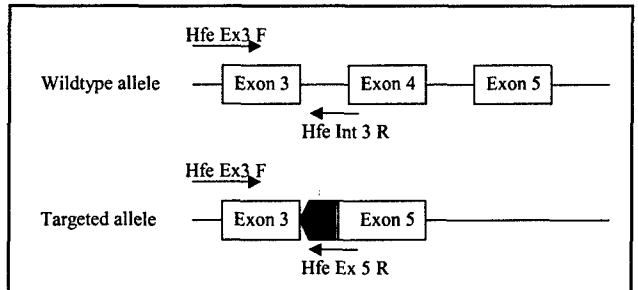

Figure 1. HFE gene knockout.

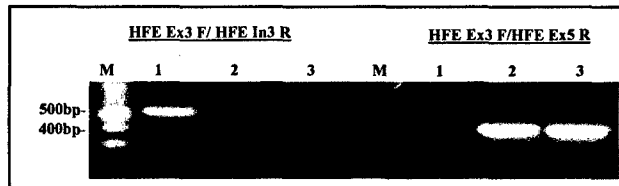

Figure 2. Genotyping of HFE mice. M: marker; 1. Wild type; 2. HFE homozygote; 3. first offspring HFE homozygote.

Genotype of HFE mice by RT-PCR:

Figure 2 shows the genotyping of the wild-type C57BL6 mouse, the original HFE homozygote, and the first offspring of HFE homozygote mice. The left panel of Figure 2 shows a band of 500 bp (primer set HFE Ex3 F/HFE Int 3 R) by the wild-type but not by HFE homozygotes. The right panel shows a band of 400 bp by HFE homozygotes but not by the wild-type mouse. It is expected that HFE heterozygotes will produce double bands (400 bp and 500 bp), though $HFE^{+/-}$ mice have not yet been bred in the laboratory and will not be used in the present study.

Evaluation of iron status in sera of wild type and iron overload HFE transgenic mice:

Serum iron (SI), serum unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and transferrin saturation (%) are determined as follows: At an acidic pH (pH 4.5) and in the presence of hydroxylamine (a reducing agent), transferrin-bound iron dissociates to release ferrous ions. These react with ferrozine to form a stable magenta-colored complex (Fe^{2+}-ferrozine) with a maximum absorption at 560 nm. The difference in absorbance at 560 nm before and after ferrozine addition in the serum sample is proportional to SI concentration. In contrast to SI, serum UIBC is measured at alkaline pH (TRIZMA®, pH 8.1). Ferrous ions added to the serum bind specifically to transferrin at unsaturated iron-binding sites and then the remaining unbound ferrous ions are measured with the ferrozine reaction. The difference between the amount of unbound iron and the total amount added to serum is equivalent to the quantity bound to transferrin, which is the UIBC. The serum TIBC equals the SI plus the UIBC. Serum transferrin saturation (%) is calculated using (SI divided by TIBC) x 100.

Table 1. SI and TS levels in wild type and HFE homozygote mice

	SI (μM)	TS (%)
Wild-type	28.6	39.1
Homozygotes	88.1	91.8

Table 1 shows the levels of serum iron (SI) and transferrin saturation (TS) in a ten-week-old wild-type C57BL6 and the first offspring of $HFE^{-/-}$ mouse at the same age. The differences in iron levels between the two mice types were striking, even though these mice were fed with the Purina base diet without iron supplementation.

Characterization of estrogen receptor status in two mouse mammary cancer cell lines:

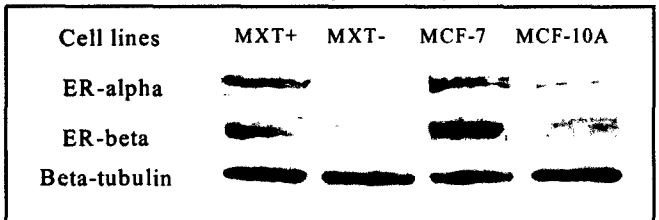

Figure 3. Western blot analysis of ER protein. Beta-tubulin was used as an internal control for protein loading. ER-beta loading is much more than ER-alpha.

MXT^+ (estrogen receptor positive and progesterone receptor positive; ER^+/PR^+) and MXT^- (ER^-/PR^+) cells are gifts from Dr. G. Bernhardt of the Institute of Pharmacy, University of Rogensburg, Germany, and are available in our laboratory (*Bernhardt, et al. 2002*). The MXT^+ cell line was derived from the murine mammary cancer model MXT^-M-

3,2 MC (hormone sensitive), which was first induced by urethane treatment in female C57BLxDBAfF1 mice as previously described (*Watson, et al. 1977*). MXT$^-$ cell line was derived from the MXT-M-3,2 (OVX) MC (hormone insensitive) mice (*Watson, et al. 1980*). Figure 3 shows the ER status of both cell lines. They were characterized in our lab by Western blot along with the human breast cancer cell lines MCF-7, known as ER$^+$, and the immortalized human breast epithelial cell line MCF-10A, known as ER$^-$. Because MXT$^+$ cells contain PR, these results indicate that ER in the MXT$^+$ cells (ER$^+$/PR$^+$) is functional, representing 55% cases in primary BC. Due to the budget constrain, only MXT$^+$ cells will be used in the present proposed research.

Changes in study design:

As originally proposed in this award, we intended to inoculate cells from the human breast cancer cell line MCF-7 into the mammary fat pads of our transgenic mouse model. However, this is not a valid approach because human cancer cells will only grow in nude mice but not in the iron overloaded transgenic mice that we have been using. We decide to use the mammary cancer cell line MXT$^+$ (ER$^+$ and PR$^+$) for the present study.

Due to the nature of the animal studies involved in this research, we are currently at the phase of feasibility tests and thus, behind the schedule as originally planned.

Key Research Accomplishments:

1) Bred and characterized the iron overloaded transgenic mice in our laboratory.

2) Evaluated the iron status in sera of wild type and HFE transgenic mice, validating our model mimicking post-menopausal conditions. Due to the cessation of menstrual cycling, iron levels in post-menopausal women are higher than in pre-menopausal women.

3) Characterized the estrogen receptor and progesterone receptor status of different human breast cancer and murine mammary cancer cell lines. Hormone replacement therapy mainly consists of estrogen and progestin, knowing the receptor status is important for data interpretation.

Reportable Outcomes:

Publication: None

Abstracts: None

Personnel:

Xi Huang, Ph.D. Principal Investigator, 10% effort

Jisen Dai, M.D. Research Associate, 100% effort.

Conclusions:

In the past year, we have performed the feasibility of various murine mammary cancer cells with different estrogen receptor status growing in our iron loaded transgenic mouse model. We are currently testing whether HRT will increase mammary tumor in these mice.

References:

Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 94:606-16(2002).

Bahram S, Gilfillan S, Kuhn LC, Moret R, Schulze JB, Lebeau A, Schumann K. Experimental hemochromatosis due to MHC class I HFE deficiency: immune status and iron metabolism. Proc Natl Acad Sci U S A 96:13312-7(1999).

Bernhardt G, Beckenlehner K, Spruss T, Schlemmer R, Reile H, Schonenberger H. Establishment and characterization of new murine breast cancer cell lines. Arch Pharm (Weinheim) 335:55-68(2002).

Levy JE, Montross LK, Cohen DE, Fleming MD, Andrews NC. The C282Y mutation causing hereditary hemochromatosis does not produce a null allele. Blood 94:9-11(1999).

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama 288:321-33(2002).

Watson C, Medina D, Clark JH. Estrogen receptor characterization in a transplantable mouse mammary tumor. Cancer Res 37:3344-8(1977).

Watson CS, Medina D, Clark JH. Characterization of progesterone receptors, estrogen receptors, and estrogen (type II)-binding sites in the hormone-independent variant of the MXT-3590 mouse mammary tumor. Endocrinology 107:1432-7(1980).

Zhou XY, Tomatsu S, Fleming RE, Parkkila S, Waheed A, Jiang J, Fei Y, Brunt EM, Ruddy DA, Prass CE, Schatzman RC, O'Neill R, Britton RS, Bacon BR, Sly WS. HFE gene knockout produces mouse model of hereditary hemochromatosis. Proc Natl Acad Sci U S A 95:2492-7.(1998).

Appendices: None